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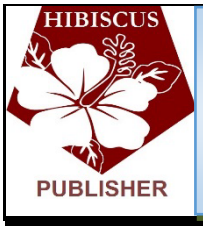


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# Preliminary Bioactivity Profiling of Methanol Crude Extract from *Clinacanthus nutans* Leaves: Antioxidant, Antimicrobial, and Apoptotic Properties

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**ABSTRACT**

*Clinacanthus nutans* (*C. nutans*), or "Belalai gajah," is traditionally used to treat microbial infections, cancer, inflammation, and diabetes due to its rich phytochemical profile. This study aimed to extract bioactive compounds from *C. nutans* leaves using varying methanolic concentrations (100%, 70%, and 60% v/v) and evaluate their total phenolic content (TPC), antioxidant, antimicrobial, and apoptotic activities. Extraction yields were 8.50%, 15.80%, and 14.70% for 100%, 70%, and 60% methanol extracts, respectively. The 100% methanol extract showed the highest TPC (23.40 mg GAE/g), followed by 70% (20.48 mg GAE/g) and 60% (20.32 mg GAE/g). Antioxidant activity assessed by DPPH assay showed the strongest radical scavenging in the 100% extract ($IC_{50}$ = 1.13 mg/mL), followed by 70% (1.87 mg/mL) and 60% (2.82 mg/mL). Only the 60% and 70% extracts exhibited antimicrobial activity with a minimum inhibitory concentration (MIC) of 10 mg/mL. Cytotoxicity assessed by MTT assay on CT26 cells revealed the lowest $IC_{50}$ for the 100% extract (0.18 ± 0.071 mg/mL), followed by 70% (0.20 ± 0.062 mg/mL) and 60% (0.49 ± 0.042 mg/mL). Apoptotic populations in CT26 cells, determined via Trypan Blue assay, were 47.34% ± 8.89, 44.37% ± 2.34, and 43.00% ± 0.95 for the 100%, 70%, and 60% extracts, respectively. These findings suggest that *C. nutans* exhibits promising antioxidant, antimicrobial, and anticancer properties, supporting its potential as a natural therapeutic agent. Further studies are needed to explore its mechanisms, dosage optimization, and synergistic interactions with other compounds.

## INTRODUCTION

Nowadays, people continue to use herbs to treat ailments because medicinal herbs have the potential to address a wide range of conditions, unlike synthetic medications, which tend to focus on specific ailments. Over the years, *C. nutans* has become a key component in various medical treatments for conditions such as cancer, diabetes, viral infections, and skin rashes [1]. *C. nutans* is a plant known by various names in different countries. In Malaysia, it is referred to as "Belalai Gajah" and "Sabah Snake Grass"; in Indonesia, it is called "Dandang Gendis" and "Kitajam" by the Javanese; and in Thailand, it is known as "Phayo Po" and "Phayo Plontong" [2]. It is primarily used and consumed through traditional methods. The *C. nutans* plant is an important species in the Acanthaceae family. Current studies estimate over 4,000 species in the Acanthaceae family, with the potential for more than 5,000 species, based on regional treatments and fieldwork that have uncovered many previously undescribed species [3]. Most species in the Acanthaceae family are tropical shrubs, herbs, or twining vines, with some also being epiphytes [4]. There are several bioactive compounds found in the *C. nutans* plant, which provide several benefits in treating certain diseases. According to Kai Xiang and Shu Xian, *C. nutans* contains saponins, flavonoids, alkaloids, terpenoids, and tannins [5].

Other studies have also identified bioactive compounds such as lupeol, stigmasterol, belutin, β-sitosterol, and myricyl alcohol. Additionally, flavonoids like schaftoside, vitexin, isovitexin, orientin, isoorientin, and isomollupentin 7-O-β-glucopyranoside were also identified in another study [4]. These bioactive compounds in *C. nutans* shows various biological activities such as antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-venom, and anti-viral properties. Antioxidants help prevent oxidative stress by maintaining a balance between oxidation and antioxidation in the body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key signals in plants, involved in metabolism, growth, stress response, and programmed cell death [6]. However, factors like cigarette smoke, alcohol, environmental toxins, and radiation can increase ROS and RNS production, disrupting this balance and leading to degenerative diseases. Exogenous antioxidants from plants can neutralize oxidative stress, slow or prevent chain reactions, remove free radicals, and quench singlet oxygen [6].

Bioactive compounds in plants inhibit microorganism growth by disrupting membranes, affecting metabolism, controlling biofilm formation, preventing bacterial capsule production, reducing toxin production, and weakening bacterial virulence through quorum sensing. Infections range from skin issues to serious conditions like pneumonia, sepsis, and meningitis. *C. nutans* leaf extract can be used externally for skin problems such as rashes, insect bites, varicella-zoster virus (chickenpox or shingles), and herpes simplex virus (HSV) lesions. It can also be consumed as herbal tea to treat fever, diarrhea, and diabetes mellitus [8]. It is also used to treat conditions such as diabetes, diarrhea, burns, skin infections, herpes, and various cancers, including lymphoma, leukemia, and tumors in the breast, cervical, colon, stomach, liver, lung, and pancreas. Cancer patients have shown recovery when consuming *C. nutans* extract orally, diluted with fruit juice or as herbal tea. Studies indicate that *C. nutans* extracts are safe and its phytochemical compounds may inhibit cancer cell growth, as demonstrated in human cancer cell lines [9]. This highlights its potential as an alternative treatment for cancer patients.

This work is meant to provide insights on the potential of *C. nutans* as an antioxidant, antimicrobial, and anti-cancer agent, and leverage it as a promising candidate for the development of plant-derived therapeutics. Particularly, this study is among the first to directly compare the bioactivity of *C. nutans* methanolic crude extracts across varying concentrations, which establishes a concentration-dependent relationship in phenolic content, antioxidant efficacy, and cytotoxicity against CT26 colon cancer cells, thus offering a more detailed foundation for future pharmacological applications.

## MATERIALS AND METHODS

### *C. nutans* Extraction

The *C. nutans* was obtained from the area of Kota Kinabalu, Sabah, Malaysia and washed under running tap water to clean the plant from dirt. The leaves were air-dried for 14 days at room temperature and then were ground using a High- speed Multifunction Grinder. The *C. nutans* powder was weighed and divided into four parts which are 10 grams each and soaked in different concentrations of methanol (100% (v/v), 80% (v/v), 70% (v/v), and 60% (v/v)) in an Erlenmeyer flask, respectively. The ratio for a mixture of *C. nutans* powder with the methanol solvent was 1:10. Then, the mixture solution was shaken using an orbital shaker (Thermo Fisher Scientific, USA) at 180 rpm for 72 hours at room temperature before filtration process. The crude extract sample was concentrated using an evaporator (Buchi R-210, Switzerland) at 56 °C. Later, the crude extract sample was weighed, and the yield percentage was calculated using the formula below:

$$Yield\ (\%) = \frac{\text{Final weight of crude extract (g)}}{\text{Initial weight of sample (g)}}\ x\ 100$$

### Total Phenolic Content (TPC) Analysis

Folin-Ciocalteau (FC) colorimetric method was used to measure the total phenolic in *C. nutans* crude extract following Raya et al.'s method with slight modification [10]. A volume of 6mL FC reagent was first diluted with dd$H_2O$ (1:1 ratio) to prepare the FC solution. Then, a serial dilution of *C. nutans* crude extract samples was prepared ranging from 5 to 0.3125 mg/mL. A 200 µL volume of *C. nutans* crude extract and standard was mixed with 1 mL of diluted Folin-Ciocalteau reagent and incubated at room temperature for 8 minutes. Then, 0.8 mL of 7.5% sodium carbonate solution was added, and the mixture was incubated for two hours. Absorbance was measured at 765 nm using an ELISA spectrophotometer. Gallic acid was used as the standard, and total phenolic content was expressed as gallic acid equivalent (GAE), calculated from the gallic acid standard curve. The total phenolic content was calculated using the following equation:

$$C = C1\ x\ mV$$

Where:
*C*: TPC in mg/g in GAE (Gallic acid equivalent)
*C1*: Concentration of Gallic acid from calibration curve in mg/mL
*V*: Volume of the extract in mL
*m*: Weight of the dry plant extract in g

### DPPH Free Radical Scavenging Activity

The antioxidant activity of *C. nutans* was determined using the DPPH assay, following previous study's protocol with slight modifications [7]. A 6 mg dose of DPPH powder was dissolved in 25 mL of methanol to prepare the mother stock solution. Ten mL of this stock was then diluted with 45 mL of methanol to achieve the working concentration. The *C. nutans* crude extract was diluted to concentrations ranging from 2 to 0.125 mg/mL. In a 96-well plate, 250 µL of the DPPH working solution was added to 50 µL of *C. nutans* crude extract. The mixture was incubated in the dark for 30 minutes at room temperature. The free radical scavenging activity was determined by the color change of the DPPH solution from purple to yellow. Absorbance was measured at 515 nm using an ELISA spectrophotometer, with methanol as the blank control. Ascorbic acid was used as the standard. The DPPH radical scavenging activity was calculated using the following formula:

$$DPPH\ radical\ scavenging\ (\%) = \frac{Ac - As}{Ac}\ x\ 100$$

Where:
*Ac*: Absorbance of DPPH in the methanol
*As*: Absorbance of sample and DPPH in methanol

### Agar Medium Preparation

The antimicrobial activity of *C. nutans* was tested using LB Agar Miller as the medium. The medium was boiled with frequent agitation to ensure complete dissolution, then autoclaved at 121°C for 15 minutes for sterilization. After sterilization, the pH of the medium was checked and found to be between 7.2 and 7.4. The medium was cooled to 40–50°C and poured into sterile petri dishes, allowing the agar to solidify. The petri dishes were then placed in airtight plastic bags and stored in a chiller at 2–8°C for future use.

**Inoculum Preparation**
Gram-negative *Escherichia coli* (*E. coli*) bacteria were obtained from the laboratory. A single colony of *E. coli* culture was picked using a wire loop and transferred into 5 mL of LB broth. The broth was incubated overnight at 37°C. The turbidity of the inoculum was then compared with the 0.5 MacFarland standard.

**Microdilution/ Minimum Inhibitory Concentration (MIC) Assay**
The minimum concentration of *C. nutans* extract that inhibits bacterial growth is recorded as the Minimum Inhibitory Concentration (MIC). The *C. nutans* crude extract was diluted to concentrations of 40 mg/mL, 20 mg/mL, 10 mg/mL, and 5 mg/mL. Then, 100 µL of each concentration was added to a 96-well plate, along with 10 µL of diluted bacterial suspension (5 x $10^5$ CFU/mL). The plate was incubated overnight at 37°C. Fresh nutrient broth without antimicrobial agents served as the negative control.

**Cell Culture**
The cell lines used were normal colon (CCD112) and colon cancer (CT26) (Lifeline Cell Technology, USA) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) medium (Nacalai Tesque, Japan) supplemented with 10% v/v, Fetal Bovine Serum (Nacalai Tesque, Japan) and 1% v/v, streptomycin/penicillin (Nacalai Tesque, Japan). The cells were incubated until 70-80% cell confluence was reached. When the cells had reached 80% confluency, the cells were passaged or trypsinized for biological assays.

**MTT Assay**
The cytotoxicity of the methanolic crude extract was measured using the IC50 value from the MTT assay [11]. The extract was serially diluted to obtain 7 concentrations ranging from 15.63 µg/mL to 1000 µg/mL to assess cell viability. A 96-well plate was seeded with 100 µL of cells (0.8 x $10^5$ cells/mL) and incubated in DMEM medium for 24 hours. After discarding the old media, 100 µL of fresh DMEM and the prepared extract were added in triplicates, followed by incubation at 37°C for 72 hours. Then, 20 µL of MTT reagent (5 mg/mL) was added and incubated for 3–4 hours. The reagent was discarded, and 100 µL of DMSO was added to solubilize the formazan crystals, with incubation for 30 minutes. The optical density (OD) at 570 nm was measured using a spectrophotometer. The cell viability percentage was calculated, and a graph was plotted to determine the IC50 using GraphPad Prism software.

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ sample}{Absorbance\ of control} \ x\ 100$$

**Trypan Blue Assay**
Cells (3.5 x $10^5$ cells/well) were seeded in a 6-well plate and incubated overnight with DMEM. After 24 hours, the media was replaced with fresh media, and 1 mL of MCECN treatment was added. The cells were incubated for 72 hours before being collected for analysis. A volume of 10 µL of the cell suspension was mixed with 10 µL of trypan blue dye. A 10 µL aliquot was loaded onto a hemocytometer and examined under an inverted microscope at 40X magnification. Viable (unstained) and non-viable (stained) cells were counted, and percentage of apoptotic cells was calculated using the formula:

$$Apoptotic\ cells\ (\%) = \frac{Number\ of\ non\ viable\ cells}{Total\ number\ of\ cells} \ x\ 100$$

**Statistical Analysis**
All experiments were performed in triplicates, and the average for each was calculated. Statistical analysis was conducted using one-way analysis of variance (ANOVA) in IBM SPSS Statistics Version 22, with data presented as mean ± standard error of mean (S.E.M) (n=3). A significance level of $p < 0.05$ was used for statistical comparisons.

## RESULTS

**Percentage Yield of Methanolic Crude Extract of *C. nutans* Leaves (MCECN)**
The leaves of *C. nutans* were extracted using the maceration method with varying methanol concentrations (100%, 70%, and 60% (v/v)). Each extract was then concentrated using a rotary evaporator. The percentage yield of each methanolic crude extract of *C. nutans* leaves was calculated. As shown in **Table 1**, the 70% methanol crude extract of *C. nutans* (70% (v/v) MCECN) exhibited the highest yield (15.80%), followed by the 60% methanol crude extract (60% (v/v) MCECN) with a yield of 14.70%. In contrast, the 100% methanol crude extract of *C. nutans* (100% (v/v) MCECN) yielded the lowest percentage (8.50%). All extractions were performed using a 1:10 ratio of dry plant weight to solvent.

**Table 1.** Percentage yield of different concentration of methanolic crude extract of *C. nutans* leaves (100%, 70%, and 60% (v/v) MCECN).

| Methanolic Crude Extract of *C. nutans* (v/v) | Initial Weight (g) | Crude Extract Weight (g) | Percentage Yield (%) |
|---|---|---|---|
| 100% | 10 | 0.85 | 8.50 |
| 70% | 10 | 1.58 | 15.80 |
| 60% | 10 | 1.47 | 14.70 |

**Total Phenolic Content (TPC) of MCECN**
The total phenolic content (TPC) of *C. nutans* leaves was determined using the Folin-Ciocalteu calorimetric method. Gallic acid was used as the standard, and a calibration curve was generated using known concentrations of gallic acid. The linear equation $y = 5.2592x + 0.0924$ was applied to calculate the TPC of each methanolic crude extract based on their absorbance values. As shown in **Fig. 1**, the TPC of 100% (v/v) MCECN was the highest at 23.40 ± 2.65 mg GAE/g, followed by 70% (v/v) MCECN at 20.48 ± 2.40 mg GAE/g, with 60% (v/v) MCECN showing the lowest TPC at 20.32 ± 2.94 mg GAE/g.

**MCECN Scavenge DPPH Radical**
**Fig. 2** illustrates the half maximal inhibitory concentration ($IC_{50}$) values for various concentrations of methanolic crude extracts of *C. nutans* leaves. The $IC_{50}$ values for ascorbic acid, 100%, 70%, and 60% (v/v) MCECN are 0.02 mg/mL, 1.13 mg/mL, 1.87 mg/mL, and 2.82 mg/mL, respectively. Ascorbic acid was used as the standard and positive control in the DPPH scavenging activity assay. Ascorbic acid exhibited the lowest $IC_{50}$ value (0.02 mg/mL), indicating the most potent antioxidant activity compared to all methanolic *C. nutans* extracts. Among the extracts, 100% (v/v) MCECN showed the lowest $IC_{50}$ value, suggesting the highest antioxidant activity. A trend of increasing $IC_{50}$ values was observed as the methanol concentration in the *C. nutans* extracts decreased from 100% (v/v) MCECN to 60% (v/v) MCECN.

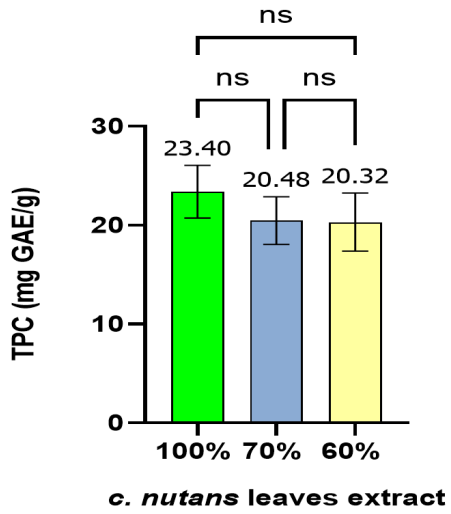


**Fig.** 1. One-way ANOVA analysis of total phenolic content of methanolic crude extracts of *C. nutans* leaves (100%, 70%, and 60% (v/v) MCECN). Results are presented as mean ± standard deviation of three independent experiments. Note: ns= not significant (p>0.05).

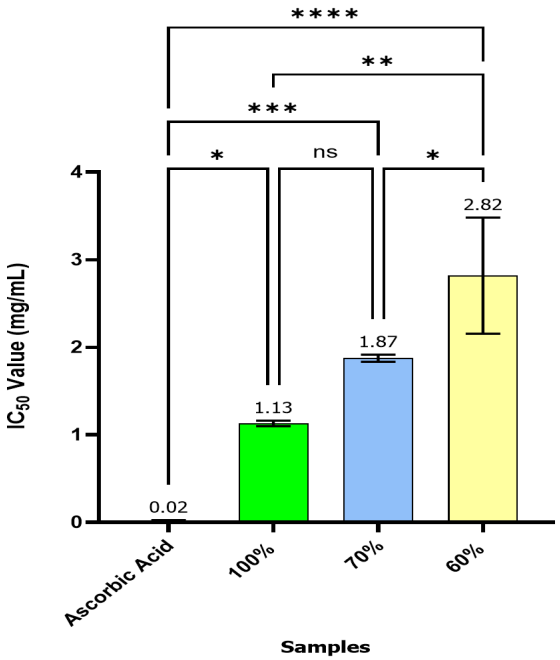


**Fig. 2**. DPPH Radical Scavenging Activity ($IC_{50}$ Values). The IC50 values of ascorbic acid (standard) and different methanolic crude extracts of *Clinacanthus nutans* leaves [100%, 70%, and 60% (v/v) MCECN]. Results are presented as mean ± standard deviation (SD) of three independent experiments. Note: ns= not significant (p>0.05), *= significant (p≤0.05), **= very significant (p≤0.01), ***= highly significant (p≤0.001), ****= extremely significant (p≤0.0001).

### Minimum Inhibitory Concentration (MIC) of MCECN against *E. coli*

**Table 2** presents the percentage of inhibition of *E. coli* growth observed at various concentrations of methanolic extracts of *C. nutans* leaves, ranging from 40 mg/mL to 5 mg/mL. The highest concentration (40 mg/mL) exhibited the most significant inhibition of *E. coli* growth across all tested concentrations. The trend in the percentage of inhibition remained consistent across the different dilutions.

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the methanol extract that can inhibit bacterial growth. The MIC is considered to be the lowest concentration of *C. nutans* extract that inhibits microbial growth by more than 95% [12]. According to **Table 2**, the MIC for both 70% (v/v) MCECN and 60% (v/v) MCECN against *E. coli* is 10 mg/mL. At this concentration, the 70% (v/v) MCECN inhibited *E. coli* growth by 95.00% ± 0.05, while the 60% (v/v) MCECN achieved 95.81% ± 0.02 inhibition. In contrast, the 100% (v/v) MCECN did not reach 95% inhibition at any tested concentration.

**Table 2.** The percentage of inhibition of *E. coli* by different concentrations of methanolic crude extract of *C. nutans* leaves (100%, 70%, and 60% (v/v) MCECN). Results are presented as mean ± standard deviation of three independent experiments.

| Methanolic Crude Extract of *C. nutans* (v/v) | Concentration (mg/mL) | Percentage of Inhibition (%) |
|---|---|---|
| 100% | 40 | 90.21 ± 0.04 |
| | 20 | 87.35 ± 0.06 |
| | 10 | 78.44 ± 0.05 |
| | 5 | 76.08 ± 0.02 |
| 70% | 40 | 100.00 ± 0.00 |
| | 20 | 97.95 ± 0.02 |
| | 10 | 95.00 ± 0.05 |
| | 5 | 84.11 ± 0.09 |
| 60% | 40 | 99.01 ± 0.01 |
| | 20 | 96.07 ± 0.04 |
| | 10 | 95.81 ± 0.02 |
| | 5 | 82.14± 0.07 |

### Anti-proliferative Effect of Methanolic Crude Extract of *C. nutans* (MCECN) on CT26 and CCD112 Cells

The cytotoxic effects of MCECN samples against the CT26 and CCD112 cell lines are presented as the percentage of cell viability in **Fig. 3**. Cells were treated with various concentrations of MCECN samples (0 mg/mL, 0.0156 mg/mL, 0.0312 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.50 mg/mL, and 1.00 mg/mL) for 72 hours. Each data point represents the mean ± standard deviation of three independent experiments. As shown in **Fig. 3**, increasing the concentration of MCECN samples resulted in a decrease in cell viability. At the 72-hour time point, the 100% (v/v) MCECN sample in CT26 cells exhibited the lowest cell viability, while the 60% (v/v) MCECN sample in CCD112 cells showed the highest cell viability at a 1.00 mg/mL concentration compared to the other samples. Additionally, the 50% reduction in cell viability for all samples in both cell lines was noted on the same graph. In the CT26 cell line, three MCECN samples: 100% (v/v), 70% (v/v), and 60% (v/v) exhibited a reduction exceeding 50%. In contrast, in the CCD112 cell line, only two samples (100% and 70% (v/v) MCECN) barely surpassed the 50% reduction mark.

In the CT26 cell line, the 100% (v/v) MCECN sample exhibited the lowest $IC_{50}$ value of 0.18 ± 0.071 mg/mL, followed by the 70% (v/v) MCECN sample at 0.20 ± 0.062 mg/mL, and the 60% (v/v) MCECN sample at 0.49 ± 0.042 mg/mL. In the CCD112 cell line, the 70% (v/v) MCECN sample displayed the highest $IC_{50}$ value of 0.96 ± 0.017 mg/mL, followed by the 100% (v/v) MCECN sample with an $IC_{50}$ value of 0.88 ± 0.009 mg/mL. As shown in **Table 3**, the $IC_{50}$ values in the CT26 cell line were consistently higher than those observed in CCD112 for all samples.

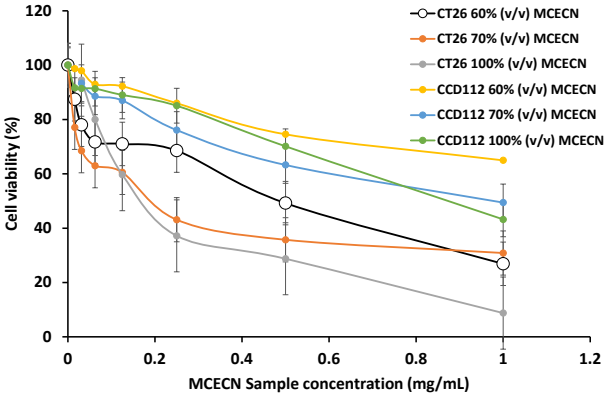


**Fig. 3.** The anti-proliferative effect of 60%, 70% and 100% (v/v) MCECN at varying concentrations (0 mg/mL to 1 mg/mL) against CT26 and CCD112 cell lines as determined by the MTT assay after 72 hours incubation. Results are presented as mean ± standard deviation of three independent experiments.

The positive control, cisplatin, demonstrated an $IC_{50}$ value of 0.44 ± 0.008 mg/mL in CT26 and 0.44 ± 0.003 mg/mL in CCD112, indicating similar cytotoxicity effects in both cell lines. Furthermore, the selectivity index (SI) was calculated as the ratio of the $IC_{50}$ value for the CCD112 (normal cell line) to the $IC_{50}$ value for the respective CT26 (cancer cell line), as shown in **Table** 3. The SI value for the 100% MCECN sample was 4.89, and the SI value for the 70% MCECN sample was 4.80, indicating nearly identical selectivity for both samples. Cisplatin, with an SI value of 1.00, is considered to exhibit general cytotoxicity across cell types. However, the SI value for the 60% MCECN sample could not be determined, as the $IC_{50}$ value for the CCD112 cell line was not observed.

**Table 3.** $IC_{50}$ value and selectivity index of different MCECN samples in CT26 and CCD112 cells 72-hours post-treatment. Results are presented as mean ± standard deviation of three independent experiments.

| Treatment Sample | $IC_{50}$ (mg/mL) | | Selectivity Index |
|---|---|---|---|
| | CT26 | CCD112 | |
| 100% (v/v) MCECN | 0.18 ± 0.071 | 0.88 ± 0.009 | 4.89 |
| 70% (v/v) MCECN | 0.20 ± 0.062 | 0.96 ± 0.017 | 4.80 |
| 60% (v/v) MCECN | 0.49 ± 0.042 | NA | NA |
| Cisplatin | 0.44 ± 0.008 | 0.44 ± 0.003 | 1 |

### Cytotoxicity Effect of MCECN on CT26 and CCD112 Cells

**Table** 4 presents the mean ± standard deviation of the percentage of apoptotic cells from three replicates of different MCECN samples in the CT26 and CCD112 cell lines. In the CT26 cell line, all three samples (100% (v/v), 70% (v/v), and 60% (v/v)) exhibited 47.34% ± 8.89, 44.37% ± 2.34, and 43.00% ± 0.95 apoptotic cells, respectively. Meanwhile, in the CCD112 cell line, the 100% (v/v) and 70% (v/v) samples showed 43.46% ± 1.08 and 43.32% ± 3.47 apoptotic cells, respectively. The negative control (no treatment) displayed the lowest apoptotic cells, with 4.00% ± 0.79 in CT26 and 3.45% ± 1.00 in CCD112. Cisplatin, however, resulted in almost similar percentage of apoptotic cells in both cell lines when compared to the MCECN treatments, with values of 44.02% ± 0.37 in CT26 and 47.21% ± 0.76 in CCD112.

## DISCUSSION

The solvent used in this study is a polar solvent, methanol, employed in various ratios with water (100% (v/v), 70% (v/v), and 60% (v/v)). Methanol is effective in extracting lower molecular weight polyphenols, which are plant compounds known for their antioxidant properties. Both methanol and water are polar solvents due to the electronegativity difference between oxygen and hydrogen (in water) and oxygen and carbon (in methanol), forming polar oxygen-hydrogen and oxygen-carbon bonds, respectively [13].

**Table 4.** The percentage of apoptotic cells in CT26 and CCD112 cell lines after treated with the $IC_{50}$ of MCECN for 72 hours. Results are presented as mean ± standard deviation of three independent experiments.

| Treatment Sample | Apoptotic Cells (%) | |
|---|---|---|
| | CT26 | CCD112 |
| 100% (v/v) MCECN | 47.34 ± 8.89 | 43.46 ± 1.08 |
| 70% (v/v) MCECN | 44.37 ± 2.34 | 43.32 ± 3.47 |
| 60% (v/v) MCECN | 43.00 ± 0.95 | NA |
| Cisplatin | 44.02 ± 0.37 | 47.21 ± 0.76 |
| Negative Control | 4.00 ± 0.79 | 3.45 ± 1.00 |

Furthermore, the structure of water allows for the extraction of other polar molecules [14, 15]. As shown in **Table 1**, the 70% (v/v) MCECN yielded the highest percentage of 15.80%, followed by 60% (v/v) MCECN at 14.70% while 100% (v/v) MCECN yielded the lowest at 8.50%. These results demonstrate that a methanol-water mixture, known as a hydroalcoholic solvent, produces a higher yield than pure methanol. The varying methanol concentrations create solvent mixtures with different polarities, which facilitate the extraction of a broader range of polyphenols, including compounds with intermediate polarities that are not fully extracted using pure methanol or water. Borges et al. have shown that a methanol-water mixture can extract a wider range of bioactive compounds, from highly polar to moderately polar, leading to higher percentage yields [16].

This study yielded a higher total phenolic content (TPC) ranging from 20.32 to 23.40 mg GAE/g (**Fig. 1**), compared to the 7 to 13 mg GAE/g reported by previous study [17]. This difference in TPC could be attributed to the different extraction methods employed. This study utilized maceration, which involves prolonged soaking of *C. nutans* in methanol. In contrast, Mustapa et al. used microwave-assisted extraction (MAE). Maceration's longer extraction time allowed for more complete extraction of a broad range of phenolic compounds compared to MAE. While MAE might not be extracting full of phenolic compounds presents in the *C. nutans* [17].

The high total phenolic content observed in this study is consistent with general trend of leaves exhibiting higher phenolic content than stem or root [18]. Yee et al. also reported that pure methanol extracts of *C. nutans* leaves showed the highest total phenolic content (23.113 mg GAE/g), compared to stem (20.933 mg GAE/g) and root (14.600 mg GAE/g) [19]. This variation in phenolic content across different plant parts is supported by Bakar et al., who stated that the different parts of the plant exhibit difference in bioactivities and chemical constituents [20]. Specific parts of plants such as leaves and roots are known to act as protective agents, responding to environmental stress by producing secondary metabolites.

This secondary metabolite will not only accumulate at the site of synthesis but will be transferred to the parts of the plants across phloem or xylem tissues [21]. It is interesting to note that plant samples of the same species collected from different geographical locations may exhibit varying biological activities. For example, Chiangchin et al. found that *Clinacanthus nutans* from different regions of Thailand showed differences in apoptotic, antimicrobial, and antioxidant activities, despite having low genetic variation [22].

**Fig. 2** displays the $IC_{50}$ values of ascorbic acid as a standard, along with different concentrations of methanolic crude extract of *C. nutans* leaves (100%, 70%, and 60% (v/v)). The $IC_{50}$ value represents the concentration of antioxidant compounds required to scavenge 50% of the initial DPPH radicals [23]. As shown in **Fig. 2**, 100% (v/v) MCECN required lower concentrations compared to the other methanolic extracts. According to Susanti et al., antioxidant activity can be assessed by the $IC_{50}$ value, where an $IC_{50}$ value less than 50 µg/mL indicates very strong antioxidant activity, and an $IC_{50}$ value between 50 µg/mL and 100 µg/mL indicates strong antioxidant activity [24]. In this study, all three MCECN had $IC_{50}$ values greater than 1000 µg/mL, indicating low antioxidant activity. This finding aligns with Alam et al., who reported that the methanol crude extract of *C. nutans* leaves exhibited low scavenging activity [4].

Minimum inhibitory concentration (MIC) is known as the lowest concentration of antimicrobial agent to inhibit the growth of specific strains of bacteria after overnight incubation. The level of MIC value can indicate that the bacterial strain is susceptible, intermediate or resistant to the antimicrobial agent [25]. This technique can help in determining the effective dosage for the potential therapeutic agent for medical uses. **Table 2** shows the minimum inhibitory concentration (MIC) of different methanolic crude extracts against *E. coli*. MIC is defined as the lowest concentration of antimicrobial agents that can inhibit 95% or greater bacterial growth [12]. The MIC for 100% (v/v) MCECN could not be determined within the tested concentration range, indicating insufficient antimicrobial activity at those levels. In contrast, both 70% and 60% (v/v) MCECN exhibited measurable inhibitory effects, with MIC values of 10 mg/mL against *Escherichia coli*. Comparatively, a study by Arullappan et al. reported that all crude extracts (petroleum ether, ethyl acetate, and methanol) and their fractions demonstrated antimicrobial activity against various microorganisms, including *Bacillus cereus, E. coli, Salmonella enterica* Typhimurium, and *Candida albicans*. Notably, Fraction 7 showed the lowest MIC and MBC/MFC values against *B. cereus* and *C. albicans*, both at 1.39 mg/mL. For reference, standard antibiotics ampicillin and amphotericin B exhibited MIC values of 1.3 mg/mL, highlighting the relatively strong antimicrobial potential of certain *C. nutans* extracts and fractions [26].

Classification by Aligiannis et al. and Mogana et al., categorize inhibition as strong (MIC<0.50 mg/mL), moderate (MIC 0.60-1.50 mg/mL), or weak (MIC>1.60 mg/mL) [27, 28]. Based on these criteria, the 70% and 60% (v/v) MCECN which exhibited an MIC value of 10 mg/mL, are considered weak inhibitors against *E. coli*. The relatively weak MIC values (10 mg/mL) observed for the 70% and 60% (v/v) MCECN against *E. coli* in this study can be directly attributed to the complex cell wall structure of the gram-negative bacteria (*E. coli*). Gram-negative bacteria have lipopolysaccharides (LPS) as their outer membrane and contain 5% to 10% peptidoglycan. This outer membrane serves as a selective permeability barrier, restricting the penetration of *C. nutans* extract into the bacterial cell due to its low permeability [28, 29]. This reduced permeability likely explains the observed weak antimicrobial activity of *C. nutans* extracts. When comparing the current study with that of Arullappan et al., it is evident that the antimicrobial activity of *Clinacanthus nutans* extracts can vary significantly depending on the extraction method and the specific fractions used. While the present study found only weak activity in the 70% and 60% (v/v) methanolic extracts (MIC of 10 mg/mL against *E. coli*) and no measurable activity in the 100% extract, Arullappan et al. reported notably stronger antimicrobial effects, with some fractions, particularly Fraction 7, achieving MIC values as low as 1.39 mg/mL. This suggests that targeted fractionation and the use of different solvents can enhance the antimicrobial potency of *C. nutans*, potentially isolating more active compounds than those present in crude methanolic extracts alone.

Recently, research has been increasingly focused on the discovery of potential anticancer drugs derived from natural sources. It is owing to the diverse bioactive compounds in the natural sources that could influence several signaling pathways and affect the proliferation of cells [30]. In this study, the cytotoxicity of methanolic crude extract from *C. nutans* leaves (MCECN) was assessed on both CT26 and CCD112 cell lines by using MTT assay. All MCECN treatments against CT26 cells exhibited different $IC_{50}$ values, as shown in **Table 3**. The 100% (v/v) MCECN showed the lowest $IC_{50}$ (0.18 ± 0.071 mg/mL), indicating the highest cytotoxic potency, followed by 70% (0.20 ± 0.062 mg/mL) and 60% (0.49 ± 0.042 mg/mL) MCECN. These variations are directly related to the differing concentrations of bioactive compounds, influenced by their solubility in the solvents used for extraction. According to Al-Yousef et al., treatments can be classified as inactive ($IC_{50}$ > 501 µg/mL), weakly active (201–500 µg/mL), moderately active (21–200 µg/mL), and highly active (≤20 µg/mL) [31, 32]. Based on this classification, the 100% and 70% MCECN extracts were moderately active, while the 60% extract was weakly active against CT26 cells. Similarly, Phung et al. reported moderately active cytotoxic effects of *C. nutans* methanol extracts against HCT116 colon cancer cells, with $IC_{50}$ values of 0.058 mg/mL (30-minute extraction) and 0.072 mg/mL (24-hour extraction) [33].

In a related study, Fong et al. investigated the cytotoxic and apoptotic effects of *C. nutans* methanol leaf extracts on D24 human melanoma cells and found that samples from higher elevations, specifically Chiang Dao and Chiang Mai exhibited the strongest cytotoxicity, with $EC_{50}$ values of 0.95 mg/mL at 24 hours and 0.77 mg/mL at 72 hours [34]. These findings collectively suggest that both extraction conditions and plant origin significantly influence the cytotoxic potential of *C. nutans* extracts. However, it is important to note that different cell types may respond differently to the same treatment due to varying cellular sensitivities, which could explain the differences observed between CT26, HCT116, and D24 responses.

The harmful effect of drug can be minimized when it specifically targets and destroys the cancer cells and prevent unnecessary injury on the healthy cells. As proposed by Artun et al., the $IC_{50}$ ratio of healthy cell to cancer cell known as selective index (SI) can be calculated to confirm the cytotoxicity of treatment. It can be used to evaluate the selective action of treatment in targeting cancer cells [35]. The findings show that the selective index (SI) of CT26 was highest when treated with 100% (v/v) MCECN (4.80), followed by an almost identical SI of 4.89 when treated with 70% (v/v) MCECN. According to Rashidi et al., the bioactive compound in the sample could develop toxicity on normal cell lines due to non-selectivity towards cancer cell lines when value less than 2 obtained from

the calculation of SI [36]. This is because normal cell line was also affected by the treatments [37]. According to Indrayanto et al., the sample can be further evaluated as a potential drug when it results in high SI value (SI ≥ 10) [38]. In drug discovery, it is crucial to develop drugs with specific concentrations that target the disease effectively while minimizing harm to normal cells [39]. It is essential to prevent cytotoxic activity towards normal cells especially in chemotherapy, as the patients may experience the adverse effect of a drug that exhibits cytotoxicity on healthy cells [40]. The findings suggest that MCECN treatments contain bioactive compounds that are safe as anticancer agents, due to their high selectivity towards the CT26 cell line.

The Trypan Blue exclusion assay results complement the MTT assay findings by providing additional insights into the cytotoxic effects of MCECN samples on both the CT26 and CCD112 cell lines. In the CT26 cell line, the 100% (v/v) MCECN sample, which demonstrated the lowest $IC_{50}$ value of 0.18 ± 0.071 mg/mL in the MTT assay, also induced a significant apoptotic response with 47.34 ± 8.89% of cells undergoing apoptosis. This suggests that the observed cell viability reduction in the MTT assay is likely linked to apoptosis. It complements the MTT assay results by providing a more direct measure of cell viability based on membrane permeability to dye. Non-viable cells retain the blue color of trypan blue due to the loss of membrane integrity, which allows dye penetration, whereas the MTT assay primarily measures mitochondrial activity. This further validates the extent of cell death induced by the different MCECN treatments [41, 42].

## CONCLUSION

In conclusion, this study demonstrated that different methanol concentrations can extract different phytochemical compounds with different biological functions (antioxidant, antimicrobial, and apoptosis properties). The findings provide a foundation for exploring the anticancer activity of the methanolic crude extract of *C. nutans* leaves, paving the way for further research into the therapeutic effects of plants in combating colon cancer. Additional studies are encouraged to identify the specific secondary metabolites responsible for the anti-microbial and anti-cancer effects of *C. nutans* leaves, to fully understand the underlying mechanisms on cancer cells.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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